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Note

Development and validation of a PCR-based functional marker system for the brown planthopper resistance gene *Bph14* in rice

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Brown planthopper (BPH) is the most damaging rice pest globally. Resistant varieties are the most effective and environmental strategy for protecting the rice crop from BPH. Functional markers (FMs) designed from polymorphic sites within gene sequences affecting phenotypic variation are highly efficient when used for marker assisted selection (MAS). *Bph14* is the first and only cloned insect resistance gene so far in rice. Compared to the sequences of its non-effective alleles there are a number SNP differences. In this study, the method of allele-specific amplification (ASA) was adopted to design a simple, co-dominant, functional marker Bph14P/N for *Bph14.* Bph14P/N was combined with two specific dominant markers: one, named Bph14P, targets the promoter region of *Bph14* and amplifies 566 bp fragments; and the other, Bph14N, targets the LRR region of *bph14* and amplifies 345 bp fragments. Specificity and applicability of the functional marker system were verified in two breeding populations and a Chinese mini core collection of *Oryza sativa*. We recommend the use of this simple, low-cost marker system in routine genotyping for *Bph14* in breeding populations.

Key Words: *Oryza sativa*, BPH resistance, allele-specific amplification (ASA), MAS, functional marker.

Introduction

The brown planthopper (*Nilaparvata lugens*; BPH) is a typical piercing–sucking pest that feeds on rice phloem sap, affecting plant growth and causing "hopperburn" (Watanabe and Kitagawa 2000). Historically considered an occasional pest of rice in tropical Asia, BPH became a severe constraint to rice production following the introduction of high-yielding varieties in the 1960s (Way and Heong 1994). According to the China Agriculture Yearbook (China Agricultural Administration 2008), there were large outbreaks in 2005–2007 with over 25 million hectares infested by rice planthopper (main BPH) populations in each year. BPH infestations have intensified across Asia and the resulting losses are considered a major reason for the fourfold increase in international rice prices since 2003 (Normile 2008). Conventional methods of controlling BPH depend on the use of poisonous chemicals and are costly in terms of labor, chemicals and the environment. Furthermore, overuse of insecticides has reduced the natural enemies of BPH and enhanced resistance of the pest to insecticides (Heinrichs and Mochida 1984). Resistant rice varieties can often interact additively or synergistically with biological control, for example bysuppressing weight gain of insects and by maintaining low BPH populations across multiple generations in a large rice production area (Cohen *et al.* 1997, Jung and Im 2005). Therefore, it is economically and environmentally beneficial to make use of host resistance (Pathak *et al.* 1969, Sogawa 1982), preferably as part of an integrated pest management strategy. Breeding for resistance is expensive since it requires facilities to undertake controlled testing. Where genetic information is available molecular marker-assisted selection (MAS) can be a highly effective breeding method because it offers rapid and precise selection of a targeted gene (Tanksley *et al.* 1989). Most studies indicate that moderate and/or polygenic resistance to insects should provide more durable resistance than single major genes (Bosque-Pérez and Buddenhagen 1992, Heinrichs 1986). To date, at least 20 genes for resistance to BPH have been identified in cultivated and wild rice species and assigned to rice chromosomes (Jairin *et al.* 2010, Jena and Kim 2010, Rahman *et al.* 2009, Yara *et al.* 2010). Among those genes, *Bph14* and *Bph15* transferred to rice from *O. officinalis*, were first

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mapped in introgression line B5 as QTLs, with *Qbph1* on chromosome 3, later renamed as *Bph14* and *Qbph2* on chromosome 4, later renamed as *Bph15* (Huang *et al.* 2001). The recent cloning of *Bph14* (Du *et al.* 2009) enables MAS of the gene in breeding programs (Hu *et al.* 2012).

Anonymous molecular markers were traditionally used to establish genetic linkage with a phenotype. However, even for tightly linked markers, the effectiveness of marker-aided selection is greatly diminished by the occasional uncoupling of the marker from the trait during the many cycles of meiosis in breeding programs. This can result in errors in selection of traits of interest (Perumalsamy *et al.* 2010). Hence, the use of functional markers (FMs) derived from polymorphic sites within gene sequences affecting phenotypic variation is more efficient for gene identification and selection (Andersen and Lübberstedt 2003). The development of FMs will become increasingly common in future, as information on cloned resistance genes becomes available. In this study, we developed a simple, codominant functional marker targeting *Bph14*. The marker was capable of being resolved on simple agarose gels and hence amenable for routine marker-assisted selection (MAS) involving large breeding populations.

Materials and Methods

Plant materials and insects

B5, an introgression line involving *O. officinalis* (Huang *et al.* 2001) was used as the source of resistance gene *Bph14*. Taichung Native 1 (TN1), a highly susceptible line to BPH was used as the negative control and B5 as the positive control in evaluations of BPH response. The BPH used for infestation were collected from rice fields in Wuchang, Hubei province, China and maintained on TN1 plants.

BPH response scoring and sorting

The bioassay was a modified bulk seedling test that followed the method of Pathak *et al.* (1969). Seeds of B5, TN1 and each group of improved lines were sown in 52 × 36-cm plastic trays. The seedlings were thinned at the three-leaf stage to 10 plants per line and infested with second to third instar BPH nymphs at a density of 10 insects per seedling with three replications. When all seedlings of TN1 had died, plants of other lines were examined and each seedling was given a score of 1 (=very slight damage) to 9 (=all plants dead) (Huang *et al.* 2001).

Development of functional markers for Bph14 alleles

Bph14 encodes a coiled-coil, nucleotide-binding, leucine-rich repeat (CC-NB-LRR) protein. Sequence comparisons indicated that the Bph14 protein carries a unique LRR domain that might function in recognition of BPH invasion to activate a defense response (Du *et al.* 2009). Sequence alignment of the LRR region of *Bph14* alleles was conducted among the four wild rice (Hy2, Hy3, Hy8, Hy9), 10 *indica* rice (B5, RI35, IR36, Babawee, Swarnalata, Pokkali, 9311, Kasalath, TN1, Zhenshan 97) and seven *japonica* varieties (02428, Balila, Hejiang 19, Nipponbare, Nongken 58, Taibei 309, Zhonghua 11). Sequence alignment of the *Bph14* promoter was conducted on B5 and *Nipponbare*. The sequence divergences were used to develop markers by designing flanking primers and then amplifying them in the rice varieties.

DNA extraction and PCR amplification

DNA was extracted from leaves following the CTAB method (Rogers and Bendich 1988) with minor modifications. Fifteen ml reaction mixtures containing 1.5 ml of PCR buffer (20 mM Tris, pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 50% glycerol), 50 ng of DNA, 330 nM of four primers each, 250 mM of each dNTP and 0.8 units of Taq polymerase. The samples were prepared in a 96 well amplification plate for amplification using a MyCycler Thermal Cycler System (Bio-Rad, USA). The PCR conditions were: 95°C for 5 min; 35 cycles of 95°C for 30 s; 58°C for 30 s; 72°C for 45 s and 72°C for 5 min. The amplified PCR products were separated on 1.5–2.5% agarose gels (Amresco, USA) stained with ethidium bromide and visualized using GelDocXR (Bio-Rad, USA). The gels were scored based on the banding pattern as positive, negative and heterozygous.

Application of the functional marker in two breeding populations

The first population (P_1, BC_1F_{10}, 9311//B5/9311) was developed by backcrossing B5 as donor to the two-line hybrid rice elite restorer line 9311 and selfing nine times to the F_{10} generation. In the backcross generation, Bph14 positive plants were selected by MAS. In the subsequent selfing generations, plants with good agronomic traits and high yield were selected.

Another population (P_2, F_6, Yihui 1577//B5/IRBB23///R6547//R105/R8) was developed by multiple crossing and then selfing five times to the F_6 generation. B5 was the donor of *Bph14*, IRBB23 was donor of the *Xa23* gene for bacterial blight resistance and Yihui 1577, R6547, R105 and R8 were elite two-line hybrid restorer lines. In the selfing generations, plants with good agronomic traits and high yield were visually selected.

Polymorphism of the functional marker in a mini core collection (MCC) of Oryza Sativa L. in China

A mini-core collection of Chinese *Oryza sativa* accessions (Zhang *et al.* 2011), obtained from Professor Li Zichao, China Agricultural University, Beijing, was genotyped to detect polymorphisms of the functional marker.

Results

Development of functional markers for Bph14

The allele-specific amplification (ASA) method was adopted to design the primer system (Bradbury *et al.* 2005).

Sequence alignment of the *Bph14* promoter was made of B5 and Nipponbare. *Bph14* shares 83% sequence identity with its allele (Os03g0848700) in Nipponbare. The coding sequence of *Bph14* in FJ941067 (accession number in NCBI) is from 2752 to 8545 and includes three exons and two introns. Comparing the upstream sequence from 1 to 2751 of FJ941067 with the -2k upstream sequence of Os03g0848700, we found four mismatched bases and an AAT repeat sequence in the 1661–1678 region and designed the forward primer Bph14PF: GGCGACTGCGAATGC TAT. We screened a special sequence in the 2207–2226 region of FJ941067, which did not have similar sequences in the Nipponbare genome and used as the reverse primer Bph14PR: GGCAGATCATCACCAACTCC (Supplemental Information). The primer pair Bph14PF/R was used for identification of *Bph14* positive individuals.

From comparison of the coding sequences of *Bph14* and its alleles, we found that the central motifs of the CC and NB domains were well conserved among diverse rice materials, but in the LRR domain 54 residues and two deletions in *Bph14* were unique. According to the specific bases, an LRR domain-specific molecular marker was designed, forward prime Bph14NF: 5′-CTACAGGCAGCCAGCAGAT-3′, reverse primer Bph14NR: 5′-TCCTGTCAGATTCTTGCA CTG-3′. Mismatch bases were introduced at the antepenultimate base of Bph14NR, to enhance the specificity and accuracy of PCR (supplementary information). The primer pairs Bph14NF/R was used for identification of Bph14 negative.

We combined Bph14PF/R and Bph14NF/R into a four-primer codominant marker system Bph14P/N. The gels were scored based on the banding pattern as positive, negative or heterozygous. When the four-primer system was used for amplification, a 566-bp fragment was amplified in *Bph14* positive plants, and a 345-bp fragment was amplified in *Bph14* negative plants. Both fragments were produced in heterozygous genotypes (Fig. 1). Because the composite primer system was designed from the gene sequence and functional region of *Bph14*, the marker is a functional marker (FM).

Verification of the functional marker in breeding populations

The BC_1F_{10} breeding population P_1 comprised 14 lines; 12 were *Bph14* positive based on the molecular testing, 10 homozygous and two heterozygous (Table 1). Homozygous positive lines were scored response 3 (MR) or lower, including 3 lines at level 0 (HR); heterozygotes had mean scores of level 5 (MS) and homozygous negative lines had mean scores of level 7 (S), the marker and phenotypic assays were in 100% agreement. Survival rates in seedling stage, the average of positive lines was than which of negative by 54%.

The more complex hybrid population P_2 comprised 125 lines, only 10 positive, including 8 homozygous and two heterozygous lines (Table 1). Positive lines had mean phenotypic scores of level 3 (MR) and the heterozygotes were level 5 (MS); Most of the negative lines were phenotypes at levels 7 (S) or 9 (HS). Survival rates from seedling stage to see, positive than the average of the negative yield increased nearly 50%.

To validate the accuracy of functional marker Bph14P/N, correlation and regression analyses were carried out. The correlation coefficient was 0.63 and P value of regression analysis is $4.48 \times$ E-15. These both showed extremely significant positive correlation between genotypes and phenotypes, indicating the FM Bph14P/N can be used to identify Bph14 accurately.

Distribution of the FM in the mini core collection

In order to further test the versatility and specificity of FM Bph14P/N, we tested the mini core collection (MCC) of *Oryza sativa* L. in China. The MCC was composed of the 200 most representative varieties, from more than 60000 accessions of Chinese cultivated rice (Zhang *et al.* 2011). One hundred and seventy four accessions amplified the negative 345-bp band, 26 produced no bands and none amplified the 500-bp positive band. The absence of positive band was consistent with the assumption that *Bph14* was not present in *O. sativa*.

Discussion

FMs developed from functional gene sequences accurately discriminate alleles at a single locus and represent ideal markers for marker-assisted selection (MAS) in breeding. FMs have apparent advantages over random DNA markers, because they are fully diagnostic of the target trait allele (Varshney *et al.* 2005). These usually neutral genetic markers can be some distance from the targeted genes, and thus are often population-specific or parent related, and their predictive value depends on the degree of linkage with the

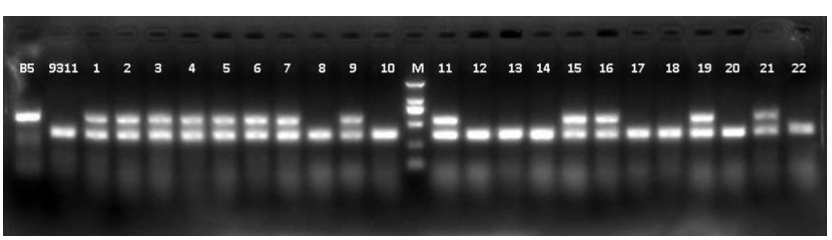

Fig. 1. PCR amplification pattern of marker Bph14P/N in a BC_1F_1 population (1.0% agrose) (M, marker DL2000: 2000-bp, 1000-bp, 750-bp, 500-bp, 250-bp, 100-bp).

Table 1. BPH response phenotypes and molecular genotypes of lines in two breeding populations

Line	seedling mortality (%)	Rating	*Bph14*[a]	Line	seedling mortality (%)	Rating	*Bph14*	Line	seedling mortality (%)	Rating	*Bph14*
						P1					
L1001	36.7	5 MS	H	L1002	36.7	5 MS	H	L1003	23.3	3 MR	+
L1004	6.7	1 R	+	L1005	70.0	7 S	–	L1006	30.0	3 MR	+
L1007	56.7	7 S	–	L1008	6.7	1 R	+	L1009	0.0	0 HR	+
L1010	0.0	0 HR	+	L1011	0.0	0 HR	+	L1012	6.7	1 R	+
L1013	13.3	3 MR	+	L1014	3.3	1 R	+				
						P2					
L2001	100.0	9 HS	–	L2002	80.0	9 HS	–	L2003	70.0	7 S	–
L2004	86.7	9 HS	–	L2005	23.3	3 MR	+	L2006	23.3	3 MR	+
L2007	23.3	3 MR	+	L2008	16.7	3 MR	+	L2009	53.3	7 S	–
L2010	90.0	9 HS	–	L2011	93.3	9 HS	–	L2012	100.0	9 HS	–
L2013	70.0	7 S	–	L2014	100.0	9 HS	–	L2015	100.0	9 HS	–
L2016	100.0	9 HS	–	L2017	100.0	9 HS	–	L2018	100.0	9 HS	–
L2019	100.0	9 HS	–	L2020	100.0	9 HS	–	L2021	100.0	9 HS	–
L2022	70.0	7 S	–	L2023	76.7	9 HS	–	L2024	16.7	3 MR	–
L2025	13.3	3 MR	–	L2026	53.3	7 S	–	L2027	30.0	3 MR	–
L2028	36.7	5 MS	–	L2029	26.7	3 MR	–	L2030	100.0	9 HS	–
L2031	100.0	9 HS	–	L2032	100.0	9 HS	–	L2033	100.0	9 HS	–
L2034	100.0	9 HS	–	L2035	100.0	9 HS	–	L2036	100.0	9 HS	–
L2037	100.0	9 HS	–	L2038	76.7	9 HS	–	L2039	90.0	9 HS	–
L2040	66.7	7 S	–	L2041	86.7	9 HS	–	L2042	90.0	9 HS	–
L2043	83.3	9 HS	–	L2044	66.7	7 S	–	L2045	63.3	7 S	–
L2046	30.0	3 MR	–	L2047	43.3	5 MS	–	L2048	70.0	7 S	–
L2049	83.3	9 HS	–	L2050	100.0	9 HS	–	L2051	86.7	9 HS	–
L2052	80.0	9 HS	–	L2053	86.7	9 HS	–	L2054	100.0	9 HS	–
L2055	100.0	9 HS	–	L2056	100.0	9 HS	–	L2057	96.7	9 HS	–
L2058	96.7	9 HS	–	L2059	56.7	7 S	–	L2060	50.0	5 MS	–
L2061	86.7	9 HS	–	L2062	76.7	9 HS	–	L2063	76.7	9 HS	–
L2064	76.7	9 HS	–	L2065	63.3	7 S	–	L2066	23.3	3 MR	–
L2067	20.0	3 MR	–	L2068	86.7	9 HS	–	L2069	100.0	9 HS	–
L2070	100.0	9 HS	–	L2071	100.0	9 HS	–	L2072	100.0	9 HS	–
L2073	100.0	9 HS	–	L2074	36.7	5 MS	–	L2075	16.7	3 MR	–
L2076	13.3	3 MR	+	L2077	93.3	9 HS	–	L2078	100.0	9 HS	–
L2079	100.0	9 HS	–	L2080	43.3	5 MS	–	L2081	10.0	1 R	+
L2082	86.7	9 HS	–	L2083	10.0	1 R	+	L2084	83.3	9 HS	–
L2085	86.7	9 HS	–	L2086	50.0	5 MS	H	L2087	66.7	7 S	–
L2088	100.0	9 HS	–	L2089	56.7	7 S	H	L2090	70.0	7 S	–
L2091	100.0	9 HS	–	L2092	100.0	9 HS	–	L2093	100.0	9 HS	–
L2094	100.0	9 HS	–	L2095	100.0	9 HS	–	L2096	100.0	9 HS	–
L2097	100.0	9 HS	–	L2098	100.0	9 HS	–	L2099	100.0	9 HS	–
L2100	100.0	9 HS	–	L2101	33.3	5 MS	–	L2102	30.0	3 MR	+
L2103	70.0	7 S	–	L2104	83.3	9 HS	–	L2105	96.7	9 HS	–
L2106	90.0	9 HS	–	L2107	100.0	9 HS	–	L2108	100.0	9 HS	–
L2109	100.0	9 HS	–	L2110	100.0	9 HS	–	L2111	66.7	7 S	–
L2112	33.3	5 MS	–	L2113	63.3	7 S	–	L2114	16.7	3 MR	–
L2115	66.7	7 S	–	L2116	16.7	3 MR	–	L2117	100.0	9 HS	–
L2118	50.0	5 MS	–	L2119	26.7	3 MR	–	L2120	13.3	3 MR	–
L2121	36.7	5 MS	–	L2122	20.0	3 MR	–	L2123	73.3	9 HS	–
L2124	73.3	9 HS	–	L2125	100.0	9 HS	–				

[a] Molecular genotypes: +, – and H indicate positive, negative and heterozygous of *Bph14* respectively.

target gene in specific populations (Bagge *et al.* 2007).

In this study, a four-primer codominant functional marker (FM) Bph14P/N was designed from specific characteristics of *Bph14*. It is the combination of the two dominant markers: one from *Bph14* upstream peculiar promoter regions, namely Bph14P; another is Bph14N, designed from differences in LRR section related to BPH response. Compared to the SSR and InDel markers used by Hu *et al.* (2012), which involve differential banding in PAGE gels and silver staining, the PCR product of Bph14P/N 566-bp or

345-bp distinguished in 1.0% agarose is simpler, faster and cheaper (Fig. 1).

BPH response is a typical quantitative characteristics in rice, and at least 20 genes for resistance to BPH have been identified and mapped (Jena and Kim 2010). Functional markers for these genes would be extremely valuable for resistance gene identification and for combining genes in breeding programs. Thus the development of a functional marker for *Bph14* in the present work appears to have been successful, but 12 lines in a complex breeding population gave low phenotypic scores but were negative for the marker (Table 1). A more likely explanation is that additional minor resistance genes were segregating and in some lines lacking *Bph14* there were enough pyramided minor genes to have a significant effect on BPH response thus confounding the results. Gene pyramiding is a powerful method of coping with a number of challenges to rice resistance and quality and has been successfully used to improve resistance to bacterial blight (Chen *et al.* 2000, Huang *et al.* 1997), resistance to blast infection (Hittalmani *et al.* 2000, Sreewongchait *et al.* 2009) and resistance to Lepidopteran insects (Jiang *et al.* 2004). Gene pyramiding, which involves the combining of two or more independent resistance genes in a particular line, offers an efficient means to cope with the BPH resistance (Hu *et al.* 2012, Sharma *et al.* 2004). However, there are few reports on pyramiding of BPH genes. The simple, codominant functional marker in this study would facilitate for MAS of *Bph14* and gene pyramiding involving large breeding populations.

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